

Title: Gene expression profile analysis as a prognostic indicator of normal tissue response to simulated space radiations

Principal investigator: Michael Story, Ph.D.
Co-investigator: David N. Stivers, Ph.D.
Grant Number: NAG9-1522
Dates: 6/1/03 – 8/31/2004
Institution: Departments of Experimental Radiation Oncology and Biostatistics
University of Texas, M.D. Anderson Cancer Center
1515 Holcombe Blvd.
Houston, TX 77030

Introduction:

This project was funded as a pilot project to determine the feasibility of using gene expression profiles to characterize the response of human cells to exposure to particulate radiations such as those encountered in the spaceflight environment. We proposed to use microarray technology to examine the gene expression patterns of a bank of well-characterized human fibroblast cell cultures. These fibroblast cultures were derived from breast or head and neck cancer patients who exhibited normal, minimal, or severe normal tissue reactions following low LET radiation exposure via radiotherapy. Furthermore, determination of SF2 values from fibroblasts cultured from these individuals were predictive of risk for severe late reactions. We hypothesized that by determining the expression of thousands of genes we could identify gene expression patterns that reflect how normal tissues respond to high Z and energy (HZE) particles, that is, that there are molecular signatures for HZE exposures. We also hypothesized that individuals who are intrinsically radiosensitive may elicit a unique response.

Because this was funded as a pilot project we focused our initial studies on logistics and appropriate experimental design, and then to test our hypothesis that there is a unique molecular response to specific particles, in this case C and Fe, for primary human skin fibroblasts.

Critical path questions:

28c: How can the individual's sensitivity to radiation carcinogenesis be estimated?

Using gene expression analysis we were to test the ability to determine whether a unique response to HZE exposure could be determined. With our model we already know that individuals can be triaged into cohorts based upon intrinsic radiosensitivity. Our presumption is that if individuals were radiosensitive they would also be susceptible to carcinogenic events. What we did not know was whether gene expression alterations were reflected in this triage process, also see 28d.

28d: How can effective biomarkers of carcinogenic risk from space radiation be developed and validated?

It would be expected that, from our model of intrinsic radiosensitivity, that biomarkers of response to radiation exposure, and perhaps to HZE exposure in particular, could be developed.

30c: How can the latency period for degenerative tissue risks, including sub-clinical diseases, following space radiation exposures be estimated?

The model chosen for this proposal is one where sub-clinical and clinical degenerative risk was already established for low LET radiations. Here, if we were allowed to continue forward we would have characterized that same risk for HZE particle exposures using our models of primary skin fibroblasts where prediction of individual late degenerative changes were already addressed.

Materials and Methods

The objectives of this project were to: 1) determine molecular profiles (gene expression patterns) for normal human skin fibroblasts that have been exposed to heavy particles of increasing LET; 2) determine if there are unique, or signature, expression patterns that define exposure to a particular HZE particle.

Our model of primary human skin fibroblasts, taken from patients being treated for either breast or head and neck cancer have been analyzed for low LET radiosensitivity and have been triaged into radiosensitive, normal, and radioresistant responses. Furthermore, documented normal tissue responses of the patient are also anonymously available and are correlated well with fibroblast SF2.

In our previous studies we showed that the range of radiation response from primary human fibroblast cultures donated by breast and head and neck cancer patients established a significant correlation between intrinsic radiosensitivity, as measured by clonogenic survival assays, and late normal tissue response. Furthermore, by using functional endpoints, we have identified specific mechanisms associated with the radiosensitivity of some cells lines. For example, two very sensitive fibroblast lines were shown to be radiosensitive because of a deficiency in DNA DSB repair (bulk rejoining), and in the fidelity of DSB rejoining.

Patients and patient samples: In the last decade we have collected, and we continue to collect, fibroblasts from head and neck and breast cancer patients. We have developed fibroblast lines from some 100 patients. A significant number of these patients have had adverse reactions to radiotherapy with documented late normal tissue reactions scored

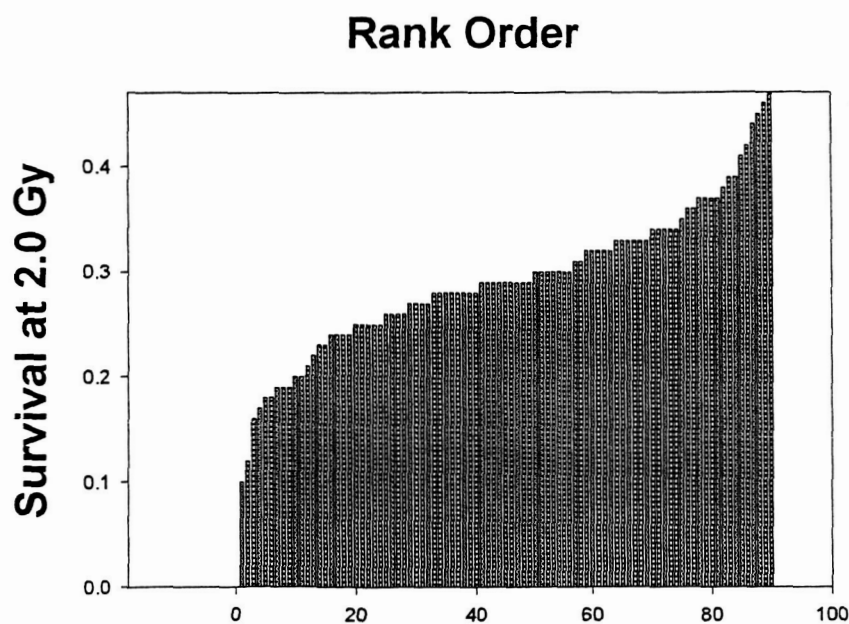


Figure 1. Rank order of fibroblast SF2 for 90 of over 100 human skin fibroblasts collected to date.

according to the RTOG/EORTC scoring system. Late effects documented include skin fibrosis, subcutaneous fibrosis, skin retraction, and soft tissue and bone necrosis. For all of these primary cultures, fibroblast SF2 values have been obtained. Figure 1 is a rank order plot showing the measured SF2 values for 90 of these fibroblast lines. (The remaining lines are lines with known genetic deficiencies, such as BRCA1/2 heterozygosity or who did not receive radiotherapy for cancer but appear to

be radiosensitive after SF2 measurements). Interestingly, SF2 ranking appears as a continuous variation which is best approximated by a normal distribution of phenotype, which can be explained by multiple interacting Mendelian factors, that is, a genetic basis for differences in SF2.

***Because this was a pilot project we limited ourselves to 5 cell lines from above, all having a normal response to radiation exposure. ***

Arrays

We selected the SigmaGenosys human oligonucleotide library which contains 18,861 oligos representing known genes and EST's. Slides were printed with a BioRobotics Microgrid II arrayer modified to use Telechem pins. In order to minimize the variability in the hybridization process and increase efficiency, we tested two automated slide hybridization systems. We chose the Lucidea SlidePro and developed a hybridization protocol based upon the Genomic Solutions protocol used to evaluate the GeneTac hybridization system with modifications similar to those developed by the Baylor College of Medicine microarray core facility. Hybridization Buffer 3 (Ambion) was used with the exception that the formamide, and hence solution viscosity, was reduced to 20% by diluting the hyb buffer 1:1 with dye-bound target cDNA in elution buffer. This solution was added at 50°C to the hyb station slide chamber which was already prewarmed 50°C. Hybridization was performed for 16 h with regular mixing. Slides were then cycled through 3 wash steps: Wash 1, 1X SSC, 0.2% SDS at 40 °C; Wash 2, 0.1X SSC, 0.2% SDS at 40 °C; and, Wash 3, 0.1X SSC at 40°C. Slides were then cycled through two isopropanol washes at RT, and then air dried.

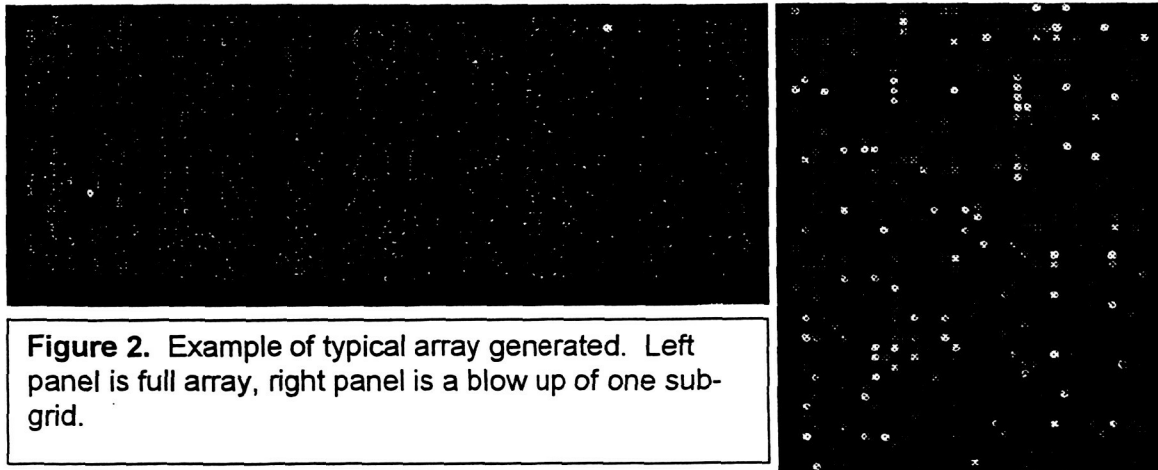


Figure 2. Example of typical array generated. Left panel is full array, right panel is a blow up of one sub-grid.

Statistical Analysis and Data Mining Strategies: All gene expression analysis was done in duplicate. Dr. Stivers, who served as co-investigator on this project, coordinated the analysis of the microarray data. Low-level microarray data analysis included quantification, normalization, quality control, and data storage. Higher order established methods included hierarchical cluster analysis, principal components analysis, and linear discriminant analysis. There were numerous established methods used to detect differential expression between two groups of samples; these methods included variants of the two-sample t-test, analysis of variance, F-test, and the Wilcoxon rank-sum test. These straightforward methods for comparing two groups using microarrays were supplemented with adjustments for multiple testing, such as permutation-based methods to estimate the family-wise error rate, total number of misclassifications (TN0oM), significance analysis of microarrays (SAM), empirical Bayes, or the beta-uniform mixture (BUM) model. Furthermore, gene ontology analysis defined pathways of differential regulation as seen below.

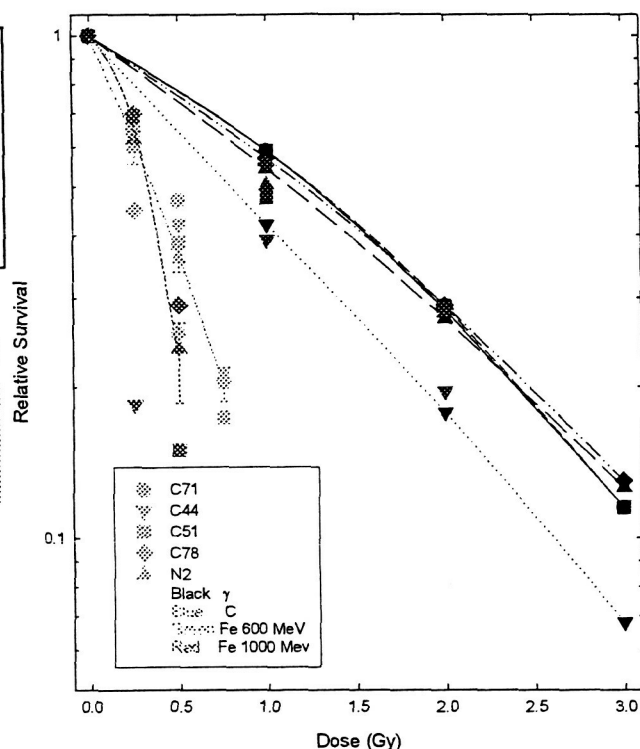
Ion Beam Selection and Time Estimates: The ion beams chosen were partially dependent on beams available. We used C, and Fe. We would examine Fe at 2 energies, 600MeV/u and 1000 MeV/u although we have not yet analyzed the 600MeV/u Fe particle data generated. Cells were irradiated as confluent cultures in T-75 flasks at a dose of 2Gy for comparison to 2Gy data from low LET experiments already performed as part of another project. The time following irradiation chosen were 1, 4, 12, and 24h after irradiation.

Results and Discussion

Our goals were two-fold. First, we wanted to develop survival plots to be able to determine RBE values for each particle in each cell line. Survival curves are shown in Figure 3 below. In the Table next to below Figure 3 are the estimated RBEs for these ions. As one might expect survival is similar for the C ions, and the difference between radiosensitive and normal still holds. However, as after exposure to Fe the differential between radiosensitive and normal is effectively gone.

Figure 3. Survival of confluent human skin fibroblast cultures irradiated with either γ -C, or Fe particles for estimation of RBEs. Particle energies, LETs, and estimated RBEs are given in the Table below.

Species	LET	RBE
¹³⁷ Cs	1	1
C (290 MeV)	13.6	1.03
Fe (577 MeV)	151.4	3.25
Fe (970 MeV)	176.1	4.44



Secondly, we wanted to develop expression signatures for HZE particle exposure. We used our 19,200 feature oligo array for these experiments. This array used the SigmaGenosys human oligo Library 1.0 and has 12,600 known genes and an additional 6,200 ESTs. We sampled the RNA at 1, 4, 12, and 24 h after a 2Gy exposure as well as sampling unirradiated cells in order to be sure our baseline gene expression did not change much between irradiations. For the most part that was true, however, there is a distinct difference in basal gene expression in unirradiated cultures processed in our home lab vs those processed at BNL. While modest, it means that if we are to go forward we must now do our low LET irradiations at BNL from now on. See Figure 4 for a view of representative data showing the cluster pattern of unirradiated samples. Red represents the reference RNA used in all experiments and it clusters together. Blue represents samples processed at BNL while green represents samples processed at our home lab.

We next examined the baseline expression of the 5 cell lines. We wanted to know if C44, the radiosensitive line had a signature that was different from the normal cell lines, C71, C51, N2, and C78. Statistical analysis revealed 45 genes that met our cutoff criteria of 1.5X fold, false discovery rate of 0.05, pvalue <0.005. Two major signaling pathways were identified by gene ontology analysis, see figure 5, and they include genes associated with DNA repair, replication, recombination, and cell growth and death. Interestingly, apoptotic pathway figures prominently. BAD and BID, and CIDE are upregulated, while BCL-2 is comparatively downregulated. This opens the door for predicting radioresponse without having to irradiate. More data are needed on other radiosensitive cells lines to validate this supposition.

The next question we wanted to answer was whether there was a distinct signature for HZE particle exposure. We chose 4 h post-irradiation as a point of interest, and in the data on the following page, figure 6, we used principle component analysis to explore the relationship between the types of radiation exposure given to all cells irrespective of their radiosensitivity.

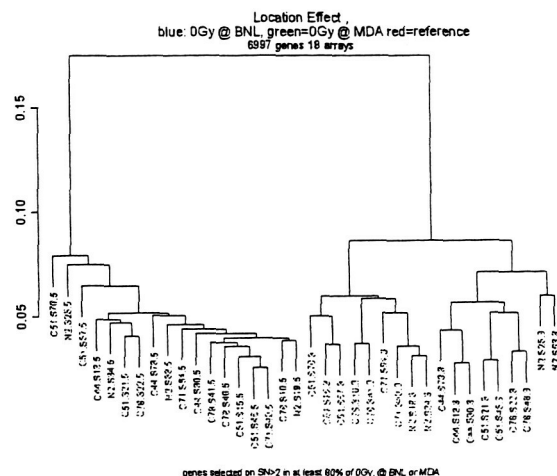
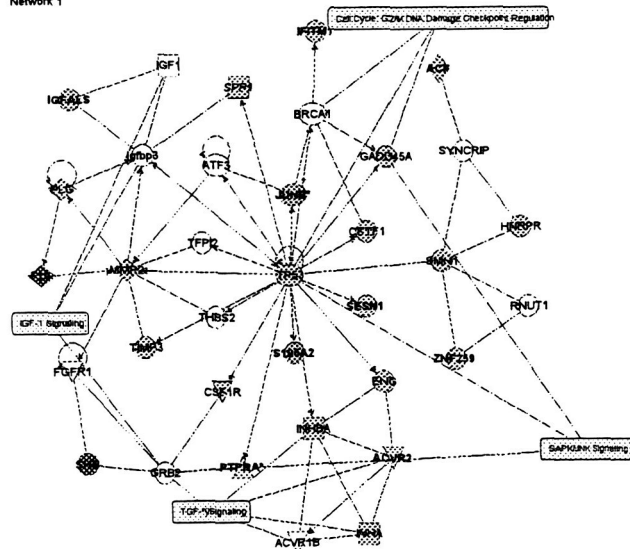


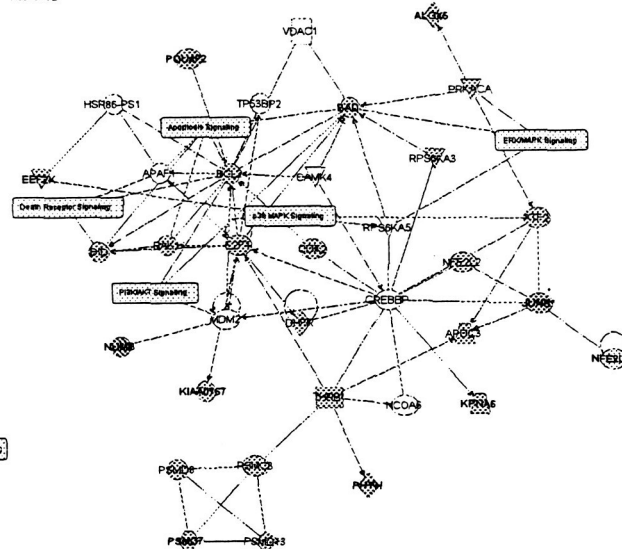
Figure 4. Unsupervised hierarchical clustering describing the location effect. Unirradiated cells have a slightly different expression profile that is dependent on their location and environment.

C44Vsotharcellines_controls_sample4: C44-all_sample4_IPA.xls
Network 1



©2000-2004, Ingenuity Systems

C44Vsotharcellines_controls_sample4: C44-all_sample4_IPA.xls
Network 2



©2000-2004, Ingenuity Systems

Figure 5. Gene ontology network analysis identifying pathways in C44 that are divergent from the 4 normal cell lines. Red represents genes upregulated in C44, green represents genes downregulated in C44, gray are not statistically different, and white represents genes in the pathway not found on the array.

While we are still processing the other samples at different time points, the 4h data shown here makes our point. These 4 images are different viewpoints, if you will, of the same data set. Consider the 4 images as rotations through a multi-dimensional space. In most of the views there are clearcut distinctions of samples based upon the type of radiation (the colored groupings are our doing and are not meant to mislead). There is the occasional outlier when viewed from a particular perspective, however, even with this small data set it makes our case that there are distinct gene expression signatures for HZE particle exposure.

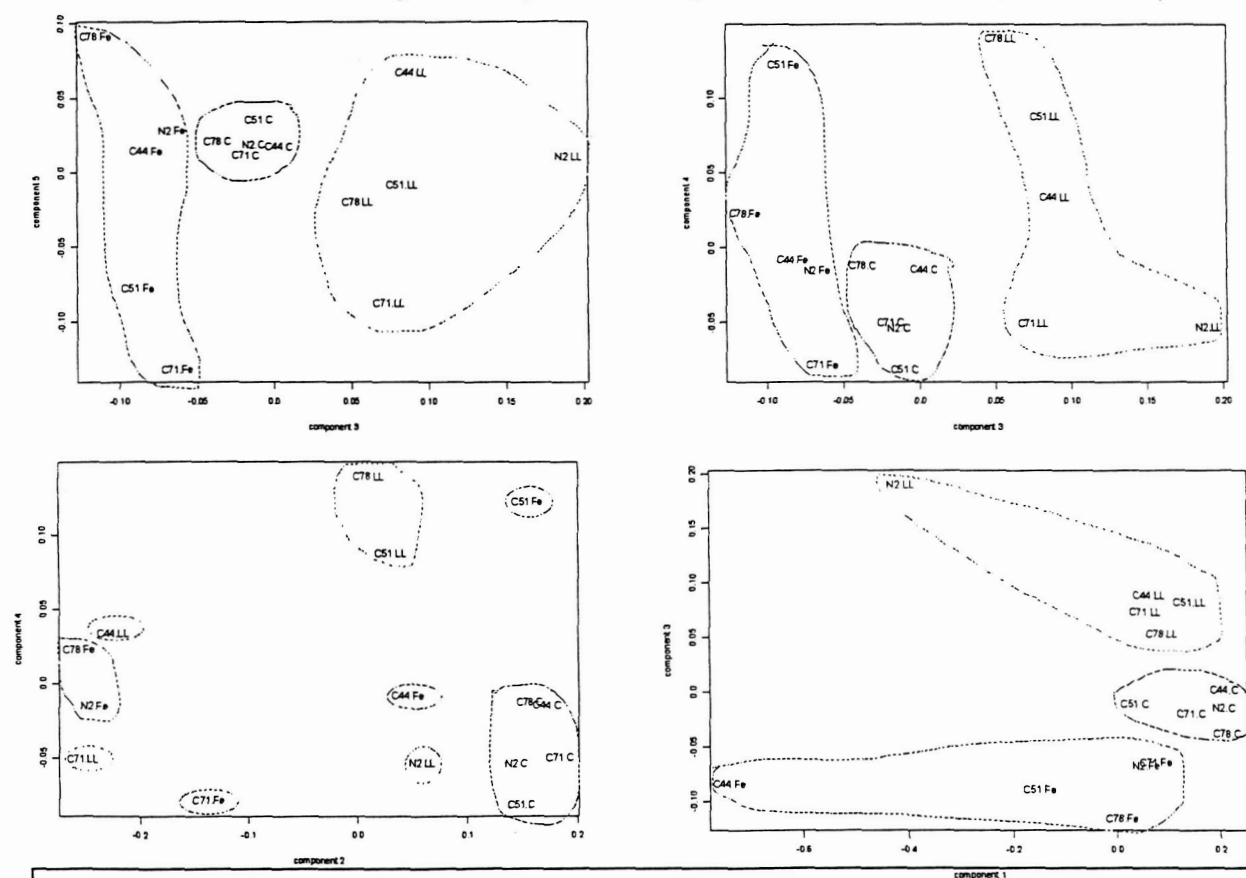


Figure 6. Principal component analysis comparing 5 cell lines based upon their response to radiation of varying type and energy 4h post-irradiation. Designations are, for example, C51Fe: cell line C51 exposed to Fe particles. LL refers to low LET γ exposure. Each panel represents a different view based upon the components compared.

When the lists of differentially expressed genes are compared (Fe vs LL and C vs LL) there is quite a bit of overlap particularly of genes associated with the cellular matrix and matrix-associated signaling. However, they are still unique from one another. There are other analyses available to us, however, space is limited and other data should be premature. Still, these data make the point. 1) Radiosensitive lines can be discriminated from normal at low LET. The discriminators that separate the radiosensitive lines may be variable and perhaps unique to each cell line. That remains to be seen. 2) there is a molecular signature that discriminates HZE from low LET exposure. There is considerable overlap in those signatures; they are, however, discernable even in small data sets. 3) These data require refinement both in terms of numbers, but in terms of other potential discriminators such as time post-irradiation. 4) These data are not at equivalent killing, at least for Fe, and that needs to be pursued, as does at least one dose caused by very low fluence to see if the signature changes.

post-irradiation. Interestingly, in this 2-dimensional plot there appears to be overlap between the unirradiated and 4h post irradiation samples. However, this is a 2-dimensional rendering of a multi-dimensional plot and by altering the orientation that overlap may not be as distinct.

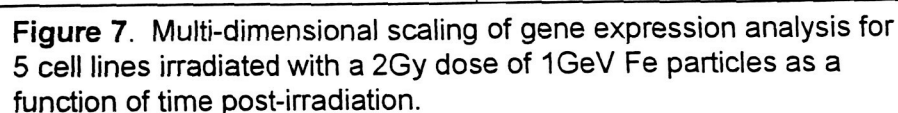
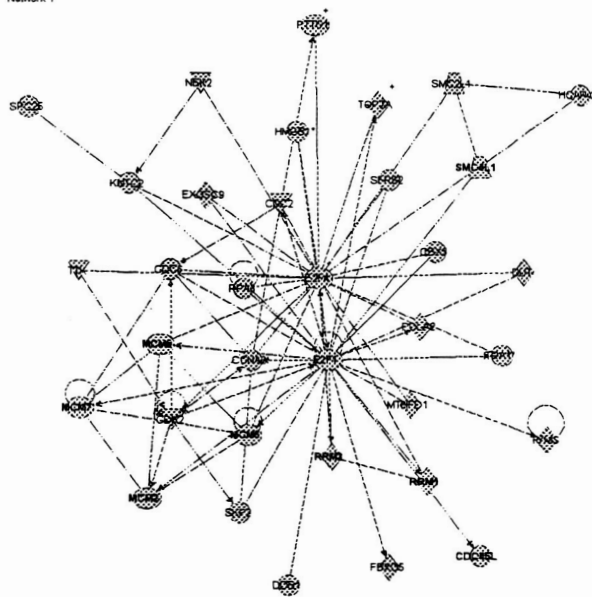


Figure 8. Top five signaling networks identified by gene ontology analysis as significantly differentially regulated 12h after a 2Gy Fe irradiation when compared to unirradiated cells. Analysis used Ingenuity (Pathways) gene ontology analysis software. Arrows refer to up or down-regulation.

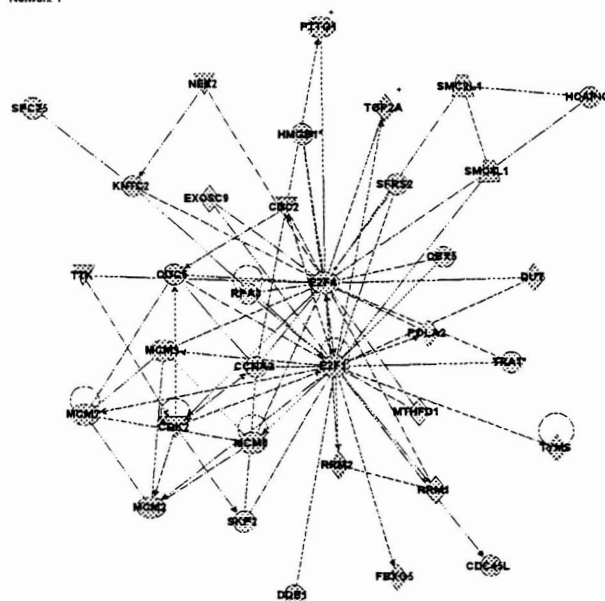
logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 1



©2000-2005, Ingenuity Systems

12 h

logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 1

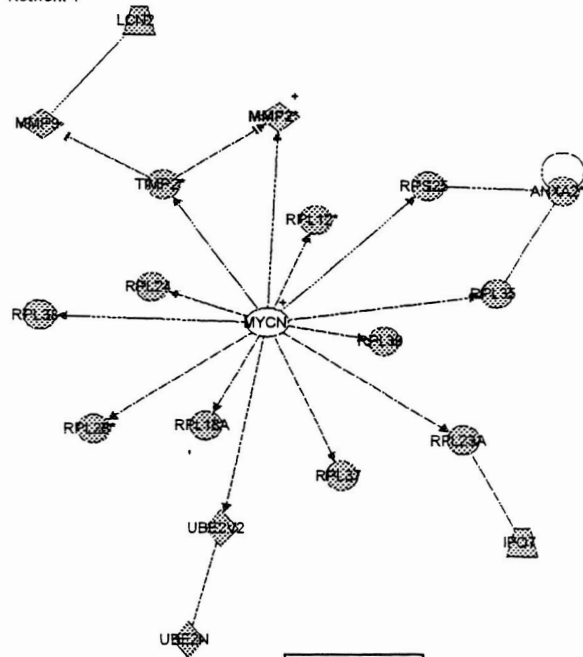


©2000-2005, Ingenuity Systems

24 h

Figure 10. Comparative gene ontology analysis of signal transduction examining the top signal transduction network seen 24h post-irradiation with the expression of those same genes 12h post-irradiation. The gene list and network descriptions can be seen in figure 9.

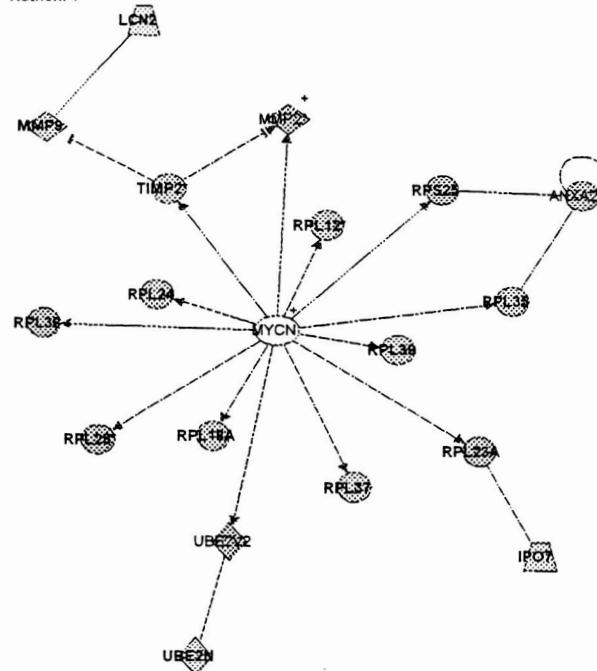
logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 4



©2000-2005, Ingenuity Systems

12h

logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 4

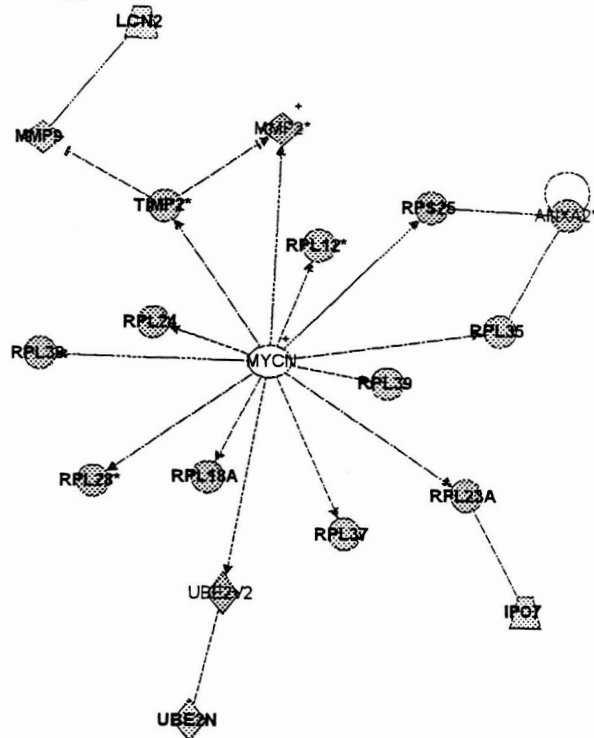


©2000-2005, Ingenuity Systems

24h

Figure 11. Gene ontology analysis for Network 4, post-translational modifications, comparing expression at 12 and 24h, based upon the differential expression patterns seen at 24h as described by Pathways (Ingenuity).

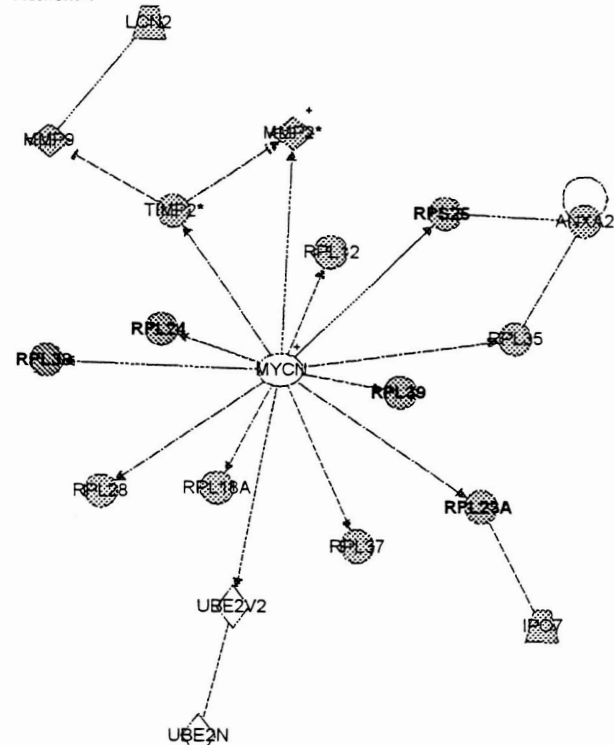
logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 4



©2000-2005, Ingenuity Systems

Fe

logratio_FE_24h: logratio_FE_24h_IPA.xls
Network 4



©2000-2005, Ingenuity Systems

Low LET

Figure 12. Comparison of gene expression after either a 2Gy Fe or γ -ray dose for genes associated with post-translational modification at 24h post-irradiation. Genes examined are those identified as differentially regulated at 24h with Fe.

It is these kinds of differences that we feel we can exploit to determine if there are unique signatures to HZE. As this was a pilot project, examining the feasibility of both the biological model and the technology used, we feel that we have accomplished our goal. There is ample evidence provided that the models and technology can be applied to the Critical Path questions listed above, as well as others associated with radiation exposure. Furthermore, we have shown that intrinsic radiosensitivity, and potentially carcinogenesis, can be examined with this technology, that there are intrinsic differences between individuals, and that gene ontology analysis shows distinct differences in the signal transduction pathways that are radiation quality, and HZE particle and energy dependent.